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Assessment of the Role of Calcium Ion in Halocarbon Hepatotoxicity

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Halogenated hydrocarbons (CCl_4, $BrCCl_3$, 1,1-dichloroethylene, bromobenzene) cause a wide spectrum of dysfunction and injury in liver cells. An early effect of CCl_4, $BrCCl_3$, and 1,1-dichloroethlylene is destruction of the Ca^{2+}-sequestering ability of the endoplasmic reticulum, and it has been suggested that this lesion leads to subsequent disruption of other cell functions. Work to test this hypothesis has begun in this and other laboratories. While it appears that redistribution of intracellular Ca^{2+} does occur following these agents, the importance of this in cell injury is not fully resolved. Current results suggest Ca^{2+} redistribution may be involved in some cases (e.g., surface blebbing caused by bromobenzene), but not in others (e.g., inhibition of lipid secretion by CCl_4).

Introduction

Halogenated hydrocarbons such as CCl_4, $BrCCl_3$, bromobenzene, 1,1-dichloroethylene, and others, are potent hepatotoxins which cause a wide spectrum of hepatocellular dysfunctions (e.g., surface blebbing, decreased lipid secretion, fatty liver, decreased protein synthesis, loss of glycogen, and ultimately, cell necrosis). It is generally considered that the hepatotoxicity of these compounds is dependent upon their metabolism by cytochrome P-450 in the mixed-function oxidase system of the liver endoplasmic reticulum (ER). In some cases (CCl_4, $BrCCl_3$) this enzyme catalyzes NADPH-dependent carbon–halogen bond cleavage with formation of highly reactive free radicals. It is usually considered that free radicals are too reactive to traverse significant intracellular distances, yet a number of cell functions remote from the ER are ultimately affected. Thus, it seems likely that some "toxigenic second messenger" must be formed which is capable of eliciting structural and functional abnormalities at distant loci. Considerable effort has been devoted to identification of this putative second messenger, and most investigations have focused on cytotoxic fragments derived from peroxidation of membrane lipids. However, as recently discussed (*1*), there is some reason to doubt that molecules of this sort are the primary mediators of haloalkane hepatotoxicity, and interest in this laboratory has turned toward another possible toxigenic second messenger, calcium ion.

The "Calcium Hypothesis"

Moore et al. (*2*) were the first to observe that an early consequence of CCl_4 poisoning in rats was destruction of the calcium sequestering activity of the ER. Because Ca^{2+} homeostasis in intact cells depends on complex and dynamic interactions between the plasma membrane, the mitochondria, and the ER, it is difficult to predict *a priori* just how loss of the Ca^{2+} sequestering system of the ER would affect intracellular Ca^{2+}. One plausible hypothesis is that as the Ca^{2+} pool of the ER leaks into the cytoplasm, a significant increase in Ca^{2+} concentration in the cytoplasm would result. If so, this elevated cytosolic Ca^{2+} concentration might be responsible for a number of pathological developments. Alternatively, Ca^{2+} leaking from the ER might be accumulated by mitochondria or transported across the plasma membrane with such efficiency that cytosolic Ca^{2+} levels might not rise significantly. If so, the depletion of Ca^{2+} from the ER *per se*, or the overloading of Ca^{2+} in the mitochondria, might lead to altered functional properties of these organelles. In any event, destruction of the Ca^{2+} sequestering activity of the ER most probably results in some redistribution of intracellular Ca^{2+}, and it seems likely that this redistribution could have significant biological and pathological consequences.

Effect of Halocarbons on Intracellular Calcium

Despite recent advances, measurement of subcellular Ca^{2+} distribution and concentration is still a difficult task, and little direct experimental evidence is available regarding the effects of halocarbons on intracellular Ca^{2+} levels. Recent measurements in this laboratory (Brattin and Waller, in preparation) have revealed that the intracellular Ca^{2+} content of hepatocytes is decreased from around 3.5 nmole/mg cell protein to around 2.3 nmole/mg by a 30-min exposure to 800 μM CCl_4. This suggests that Ca^{2+} released from the ER is

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transported out of the cell by the activity of the plasma membrane Ca^{2+}-ATPase. This observation is in contrast to several reports that CCl_4 administered *in vivo* results in a severalfold increase in liver Ca^{2+} content (*3,4*). Such an increase cannot easily be explained by an effect of CCl_4 on the ER, and suggests that Ca^{2+} balance at the plasma membrane is disturbed. Several reports of CCl_4-induced inhibition of the hepatic plasmalemmal Ca^{2+}–ATPase have appeared (*5,6*), but this effect does not appear to require metabolism of CCl_4, and most likely is the result of "solvent effect" on the plasma membrane. In support of this, we find that high doses of CCl_4 (1.5 mM or higher) do cause net accumulation of Ca^{2+} in isolated hepatocytes.

Of special interest is the effect of CCl_4 and related compounds on the concentration of free Ca^{2+} in the cytoplasm of hepatocytes. We are investigating this issue using the cytoplasmic Ca^{2+} indicator "quin-2" (*7*). Preliminary findings suggest that exposure of isolated cells to 1 mM CCl_4 results in a rapid (30–60 sec) increase in free cytoplasmic Ca^{2+}. The magnitude of the increase is similar to that evoked by 10^{-6}M phenylephrine (*8*). Whether this CCl_4-induced increase in cytosolic Ca^{2+} is a consequence of Ca^{2+} release from the ER or of altered plasma membrane permeability to Ca^{2+} is not yet clear.

Role of Altered Calcium Distribution in Cell Dsyfunction

Jewell et al. (*9*), employing a spectrophotometric assay using arsenazo III as a Ca^{2+} indicator, found that surface blebbing in hepatocytes following bromobenzene exposure correlated with a decrease in extramitochondrial Ca^{2+} (a pool which probably consists primarily of ER Ca^{2+}). We have examined the effects of bromobenzene administration to rats on ER calcium sequestration, and found that a dose of 800 μL/kg produces a 28% decrease in ER Ca^{2+} sequestration measured 24 hr later. Whether this decrease could account for the loss of extramitochondrial Ca^{2+} seen by Jewell et al. is not known. Interestingly, Jewell et al. were able to mimic bromobenzene-induced surface blebbling by incubation of hepatoctyes with A23187 in the absence but not in the presence of extracellular Ca^{2+}. This suggests that Ca^{2+} depletion from some intracellular site is responsible for surface blebbling, rather than an elevation in cytosolic Ca^{2+}.

Another prominent hepatic dysfunction following CCl_4 poisoning is inhibition of VLDL secretion (*10*). We have sought to determine whether Ca^{2+} might be a mediator of this dysfunction. VLDL secretion by isolated hepatocytes is inhibited much more rapidly after CCl_4 treatment than is the Ca^{2+} sequestering activity of the ER (*11*). Also, the dose-response curve for CCl_4-induced loss of VLDL secretion is left-shifted (about 2-fold) from that of ER calcium sequestration. This lack of correlation suggests, but does not prove, that destruction of the Ca^{2+} sequestering system of the ER is not required for the CCl_4-dependent inhibition of VLDL secretion. Incubation of hepatocytes with A23187 in the presence of various extracellular Ca^{2+} levels did not result in inhibition of VLDL secretion (*11*). Taken together, these observations suggest that disturbances in intracellular Ca^{2+} may not be an important step in CCl_4-induced inhibition of VLDL secretion.

Role of Extracellular Calcium

While the total Ca^{2+} content of liver rises significantly within 1 hr of exposure to CCl_4, there is a much larger rise about 12 to 24 hr after poisoning (*12*). This late increase in liver calcium is thought to reflect a decrease in the integrity of the hepatocyte plasma membrane with concomitant influx of Ca^{2+} from the medium. Absence of Ca^{2+} in the medium during this time has a protective effect, diminishing cell injury as judged by trypan blue exclusion (*13*). However, influx of extracellular Ca^{2+} does not appear to be required for the earlier injuries to the cell. For example, experiments in this laboratory have shown that CCl_4-induced inhibition of VLDL secretion is essentially unaffected by the absence of extracellular Ca^{2+} (*11*). Similarly, Smith et al. (*14*) reported that several hepatotoxins (including CCl_4 and bromobenzene) were actually more toxic to hepatocytes in the absence than in the presence of extracellular Ca^{2+}. It should be recognized that the Ca^{2+} stores of the ER may be important in maintaining normal cytoplasmic Ca^{2+} levels in the face of reduced extracellular Ca^{2+}, and thus destruction of the ER Ca^{+} pool by halocarbon poisoning might be expected to cause increased cell sensitivity to exposure to low extracellular Ca^{2+}.

Summary and Conclusions

Exposure of hepatoctyes to CCl_4 and certain other halocarbons results in rapid destruction of the Ca^{2+} sequestering activity of the endoplasmic reticulum. *A priori*, it seems likely that a major redistribution of intracellular Ca^{2+} will occur as a consequence, and it is possible that this Ca^{2+} redistribution has pathological effects.

Results from this laboratory reveal that low doses of CCl_4 cause Ca^{2+} loss from hepatoctyes, but the intracellular origin of this Ca^{2+} is not yet certain. Exposure of isolated cells to CCl_4 also results in a rapid increase in free cytoplasmic Ca^{2+}, but, again, the reason for this increase is not yet clear. Whether such halocarbon-induced changes in cell Ca^{2+} are responsible for any aspect of cell injury is unknown. Regarding the phenomenon of bromobenzene-induced surface blebbing, there is suggestive evidence that Ca^{2+} depletion from the cell might be causal. With regard to CCl_4 -induced inhibition of VLDL secretion, the evidence suggests that altered Ca^{2+} distribution may not be essential. Influx of Ca^{2+} from the medium does not appear to be involved in either of these two processes, but may be important in the development of later cell pathology. Thus, Ca^{2+} may play different roles in the development of various aspects of cell injury, and considerable work remains to be performed in order to resolve this fascinating aspect of cellular toxicology.

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